REMARKS

The Examiner has rejected Claims 3-10, 12-17, 23-25, 27, 28-41, 42, 43 and 44-51 based on informalities. Specifically, these claims were dependant upon Claim 1, which was previously cancelled. This has been corrected by amending all claims previously dependant on Claim 1 to be dependant on Claim 2 or another intervening claim. The Examiner has also objected to the use of the phrase "can be" in Claims 28, 48, 50 and 51 as being indefinite. In response, the Applicant has modified Claims 28 and 48 to remove the offending phrase and has deleted Claims 50 and 51 from the application.

One difficulty experienced by the Applicant in the prosecution of this application is the meaning of the various terms used to describe the functions of the fiberscope and their consistent use in the application, by the Examiner and in the prior art cited references. In particular, the Applicant believes there is a misinterpretation on the part of the Examiner and a difference in meaning between the prior art and the present application with respect to the use of the terms "Raman chemical image", "Raman chemical map " and "Raman spectroscopy". As an aid in understanding the features which distinguish this application from the prior art, the Applicant offers the following definitions of terms used in the application and herein.

Raman Spectroscopy: Measurement of spectral features of an object wherein the Raman effect is responsible for the energy shifts which give rise to the spectral variability which is observed.

Raman Chemical Map: A map wherein the information being depicted is the chemical constituents as determined from some observation of the Raman effect which is generated by acquiring Raman spectra from a plurality of points on a point-by-point basis, as a focused laser beam is scanned across the surface of the object being sampled, or, more commonly, by moving the laser in a raster scanning fashion and collecting Raman spectra at each spatial location.

Raman Chemical Image: A two dimensional, lateral spatial depiction of the chemical constituents of an object wherein the spatially resolved depiction of the chemical constituents is derived from the observation of Raman shifted

light recorded in a spatially resolved whole field of view in a single measurement, generated by acquiring high resolution images of the sample at discrete wavelengths of light which correspond to specific energies (wavelengths) in the Raman spectrum.

Raman spectroscopy, as is well known in the prior art, involves illuminating a small region of a sample with light, collecting the scattered Raman light and spectrally analyzing it. Also well known in the prior art is the creation of a Raman chemical map of the sample under study. Those of skill in the art will recognize the typical procedure for the creation of a Raman chemical map as follows. First, the excitation light source is placed over one spatially-defined point of the surface of the sample. Second, Raman shifted light emanating from that point is collected. Third, the probe is then moved to a plurality of other spatially-defined points, where the first and second steps are repeated. This collection of Raman spectra is typically done in a raster scan manner over a much larger region than any of the individual points alone. Each of the points sampled has associated with it a Raman spectrum. The Raman spectra at all of the different points are then used to create a map of the different chemical features over all of the regions where the samples were collected. Generation of a Raman chemical map can clearly be performed using the fiber optic device disclosed in the cited Wach reference to map out the different spectra originating from various points on the surface of the sample. This requires, however, that spectra from a plurality of points be sequentially acquired by moving the probe from point to point, and that a sufficient plurality of points exist to produce images of sufficiently high resolution (i.e., high fidelity) to observe features and variations in the Raman spectra over the sample.

The Raman chemical map which is well known in the art would appear similar to a Raman chemical image, as taught by the present application. However, the Raman chemical image of the present application is derived in a technically novel way. The Raman chemical imaging approached developed by the Applicant and described in the current application allows all points in an image (currently up to 256,000 pixels) to be simultaneously acquired and analyzed to obtain Raman spectra at a plurality of spatially-defined pixels (i.e., points on the surface of the sample) during a single measurement/acquisition period by using a coherent fiber bundle as the spectral collection media and a full field spectral detector capable of both the high spatial resolution required for imaging and the high spectral resolution required for Raman spectroscopy. High fidelity lateral resolution is essential for the practical use of any chemical imaging system. The level of resolution produced by the Applicant's invention is demonstrated in

Figures 3, 4, 8 and 9 to be sufficient to produce highly resolved images that are not pixilated and which show clearly-resolved features. Figure 4 is a standard resolution calibration grid that indicates the high resolution achieved. Effectively, this single scan spectral imaging allows massive parallel processing of the spectral information from different regions of the sample at the same time during a single measurement. This is in contrast to the slower point-by-point spectral acquisition required by the Wach or Alfano references of the prior art.

Due to the complexities of fiber optics transport and scattering, as taught by Wach, one skilled in the art would not expect to be able to preserve both the spatial and spectral information at the same time, which has led to work using a mapping technique, as would be used by the device disclosed by Wach. However, preserving both the spatial and the spectral information is precisely what is needed to acquire a Raman chemical image with a single Raman data acquisition, as opposed to a Raman chemical map.

The unique filters and optical arrangements provided in the current application demonstrate and teach how full fidelity, one shot spectral imaging can be done, contrary to previous teachings by both Wach and Alfano. In the current application, the fiberscope claimed can perform three functions, all through the same coherent fiber bundle. First, Raman spectra can be collected at multiple points on the surface of the sample without moving or repositioning the fiber. Second, a Raman chemical image can be obtained in a single acquisition when the sample is illuminated by a laser light and third, an optical image can be obtained of the surface of the sample when the sample is illuminated with white light. Therefore, the fiberscope of the current application provides fibers for the delivery of white light from a white light source, fibers to deliver laser light to the sample from a laser source and a coherent fiber bundle for collecting white light images, Raman spectra and Raman chemical images. Additionally, the claims include optics and filters needed to filter and focus the light delivered to the sample and light collected from the sample.

In paragraph three of the Office Action, the Examiner rejects Claims 2, 18, 20, 28, 29-35, 44, 48 and 49 under 35 U.S.C. § 102(e) as being anticipated by U.S. Patent 6,222,970 (Wach, et al.). Wach describes how Raman spectra provides chemical information and how, by using an appropriately designed optical fiber and proper filtering, one can collect high quality Raman spectra remotely from the sample. This involves taking a Raman spectrum at a single point by illuminating that point through the fiber, collecting Raman scattering at the sampled point via the fiber and transporting the Raman scattered light to a remote point where it is analyzed. Wach's disclosure (Columns 1-13) discusses all of the limitations and problems related to obtaining a Raman spectrum from a single point through the fiber. The single point that Wach refers to is

strictly one region of the surface of the sample. One skilled in the art would interpret this as remotely collecting a Raman spectrum of a single point on the surface of the sample.

Raman is a very difficult technique with very weak signals. Wach states, in column 1, line 66 through column 2, line 8 that "the resulting low light levels require sophisticated, expensive instrumentation and technical complexity." The Wach reference teaches how to optimize a plurality of fibers for performing Raman spectroscopy and various designs to do so. His specification and designs referring to a plurality of filters are meant to optimize the collection efficiencies to achieve a Raman spectra at a remote point from the sample through a plurality of fibers. One skilled in the art would not be able to use his teachings to create a device utilizing a coherent fiber bundle that can perform both remote Raman spectroscopy and Raman chemical imaging, as well as white-light optical imaging.

When one talks of using a coherent fiber bundle, one usually assumes transport of an image. However, Wach explicitly states that the use of fiber bundles introduces additional problems and undesirable characteristics. See column 9, lines 7-9. Therefore, Wach describes ways to optimize the collection of the spectra which focuses on small numbers of fibers. With respect to the term "coherent fiber bundle," this is a well known term of art to those of skill in this art. The term coherent fiber bundle infers a bundle of fiber strands, each one carrying a point (pixel) of an image. For imaging, the fiber bundle must be coherent, which means that the ends of the fibers must be arranged in the same way on both ends of the bundle of fiber strands. Coherent fiber bundles are discussed in detailed in Chapter 28 of the book "Understanding Fiberoptics" 3d Edition by Jeff Hecht, a copy of which is attached to this response and with which the Examiner is already familiar. Those of skill in the art understand that small numbers of fibers do not provide the fidelity to spatially resolve physical structure or features in an image. From Wach's cited complications regarding using fiber bundles, and the forms of the fibers which Wach discloses and shows in his figures, he teaches away from using large numbers of fibers. This is also true of the Alfano reference. Further, neither Wach nor Alfano shows any evidence that a high fidelity optical image could be obtained using any of the configurations disclosed, which are needed to perform Raman spectroscopy. This is in contrast to the present invention, which demonstrates both the ability to obtain high fidelity optical images and single scan Raman chemical images using the same fiber optics assembly.

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The only reference made by Wach to a fiber bundle is in the context of the instrument interface at the remote viewing point as discussed in column 36, lines 19-49. This is used to transport the signal passing out of the fiber into the analysis instrument. He notes that bundles are "used for imaging applications" but describes their use for his purposes in the context of

creating an adapter to transfer circular Raman outputs (signal) into a line, or slit style form to allow a circular beam of Raman light coming from the end of the fiber at the remote viewing point to be efficiently focused on the physical linear slit of a spectrometer, which is used to analyze the scattered light. The slit configuration provides for higher efficiency and better spectral signals. The reference to imaging made by Wach, cited by the Examiner, is in the context of providing an historical reference for the use of fiber optic bundles. However, the configuration disclosed in Wach cannot be described as a "coherent fiber bundle" (which would be capable of transmitting a high fidelity image) as the term is understood by those with skill in the art, simply because the individual fiber strands in the bundles are not arranged in the same manner on the collection and viewing ends of the fiber bundle. Therefore, it is not possible to view an image through the bundle described in the Wach reference . Additionally, it is the Applicant's position that the number of fibers in the Wach "bundle" is insufficient to transmit a high fidelity image. The Applicant therefore respectfully submits that Wach teaches away from trying to preserve a high fidelity optical image of the surface of the sample but, instead, concentrates on various methods to preserve the *spectral quality* of the Raman scattered light and Raman spectra as it is transported into and through the fiber and then analyzed at the remote viewing point. Wach discloses the collection of spectral data from a single region and does not refer to or use the term "Raman chemical image" or any equivalent. These differences clearly distinguish the present invention from the Wach reference.

In response to the rejection of the claims under § 102(e) over Wach, the Applicant has modified the claims in question to make it clear that video (i.e., optical images), Raman chemical images and Raman spectra are collected through the single coherent fiber bundle of the present invention and respectfully submits that this is not disclosed by Wach. First, as discussed, Wach does not disclose a coherent fiber bundle. Second, Wach discloses the collection of Raman spectra at single points on the surface of the sample, not at a plurality of points simultaneously, which could then be combined to form a Raman chemical map, but not a Raman chemical image. Additionally, the Wach reference does not mention optical imaging. Furthermore, Wach does not disclose an outer jacket containing a coherent fiber bundle, a plurality of white light illumination fibers and a plurality of laser illumination fibers.

The Examiner has further rejected Claims 10, 12-17, 21, 22, 27, 42, 50 and 51 under 35 U.S.C. § 103(a) as being unpatentable over Wach, et al. in view of U.S. Patent 6,091,872 (Katoot). The Examiner states that all the limitations of the claims are disclosed in Wach except the plurality of white light illumination fibers for transmitting white light from a second source to the sample. The Applicant respectfully submits that, although Katoot teaches using white

light illumination fibers in combination with fibers for the transmission of other types of light, such as laser light, Katoot does not provide a coherent fiber bundle within the same jacket as the white light and laser light illumination fibers for the collection of Raman spectra, Raman chemical images and optical images. Additionally, no combination of Wach or Katoot teaches all of the limitations of the present invention as claimed.

The Examiner has further rejected Claims 23 and 45 under 35 U.S.C. § 103(a) as being unpatentable over Wach in view of U.S. Patent 6,006,001 (Alfano, et al.). The Examiner states that Wach teaches the invention but does not teach a mounting for holding the fiberscope end in proximity to the sample, a link for directing the output of the fiberscope under white light illumination conditions to a live video camera, a link for directing the output under laser illumination conditions to a Raman spectrometer and a link for directing the output under laser illumination conditions to a Raman chemical imaging spectrometer and detector. The Examiner states that Alfano teaches these features of the claimed invention. The Applicant respectfully submits that Alfano differs from the current invention in the following ways. First, Alfano teaches the use of a scope containing illumination fiber 27 and plurality of collection fibers 29 as shown in Figure 2 and Figure 4, collectively referred to as reference number 21. In addition, in Figure 6, the Examiner states that Alfano discloses a mount 30 for holding the fiber scope distal end in proximity to the sample. However, 30 is clearly referred to in the specification as a tip placed inside the working channel of the endoscope and mounted on the end of the bundle 52 to reach certain parts of the body in vivo. See column 8, lines 25-30. Further, note that Alfano also does not teach a fiberscope having a plurality of white light illumination fibers, a plurality of laser illumination fibers and a coherent fiber bundle through which Raman spectra, Raman chemical images and optical images are collected. Figure 6 of Alfano clearly shows endoscope 73 having fiber scope 21 inserted therein wherein fiber scope 21 has laser illumination fibers and a plurality of collection fibers as previously described. However, the unreferenced objects in endoscope 73 are in all likelihood a white light illumination fiber and a coherent fiber bundle for the collection of optical images from the sample. Clearly, in Alfano, there are no Raman chemical images. Furthermore, Raman spectra and optical images are not collected through a single coherent fiber bundle and the fibers linked to the spectrometer cannot be described as a coherent fiber bundle. Further, Claims 23 and 45 have been amended to include a link for directing the output of the fiber scope under laser illumination conditions to a liquid crystal tunable filter imaging spectrometer. Such a link is not disclosed in Alfano.

The Examiner has further rejected Claims 24, 25, 46 and 47 under 35 U.S.C. § 103(a) as being unpatenable over Wach in view of Alfano, et al. and further in view of a paper entitled

Remote Raman Micro imaging Using An AOTF and Spatially Coherent Micro Fiber Optical Probe, Cooney, et al, Applied Spectroscopy, vol. 50, no. 8 (1996). With respect to the Cooney reference, the Examiner states that it would have been obvious to one of ordinary skill in the art to utilize a liquid crystal tunable filter spectrometer in place of the AOTF, as stated at column 1, lines 25-33 of Cooney. However, the Applicant respectfully submits that Cooney, as well as the references labeled 14 and 15 cited in Cooney, teach away from the use of a LCTF for the purpose of Raman Chemical Imaging, due to severe limitations in the technology at the time of the publication of the references. References 14 and 15 cited by Cooney demonstrate that the art of liquid crystal tunable filters at this time was not adequate to satisfactorily perform Raman chemical imaging.

Reference 14 of Cooney, *Imaging Spectrometers for Fluorescence and Raman Microscopy: Acousto-Optic and Liquid Crystal Tunable* Filters, Morris, et al., Applied Spectroscopy, Volume 48, Number 7 (1994) states as follows:

> the LCTF provides a broad bandpass that is not sufficient for resolving individual Raman bands...

and further:

> The LCTF employed in this study is not optimized for Raman imaging because of the low transmission and the course spectral resolution. For optimized Raman imaging performance, a high resolution (5 cm^{-1}), high transmission device would be suitable, but at a cost to the free spectral range of the device.

Id. at 865.

A suitable free spectral range is necessary in a Raman Chemical imaging device because of the wide range of materials which may be detected on the surface of the object being sampled. Ideally, the free spectral range of the device would cover the range of about 500 cm^{-1} to 3500 cm^{-1}. At the time of the Cooney reference cited by the examiner, it was unknown how to provide a LCTF having high resolution, high tranmissivity and a large free spectral range.

Likewise, Reference 15 of Cooney, *Ultrasensitive Raman and Flourescence Imaging Using Liquid Crystal Tunable Filters*, Morris, SPIE, Volume 2385 (1995) states as follows:

> The demonstrated Lyot filter has these liquid crystal filter advantages, although the pass band of about 200 cm-1 is too wide for almost all Raman imaging. The Lyot pass band can certainly be reduced, but its inherently low transmission remains a serious limitation of this technology.

Id. at 80, agreeing with the assessment of Reference 14. This paper goes on to discuss an alternate interferometer for Raman imaging applications, (i.e.. a Fabry-Perot interferometer) which the author further indicates is inadequate for Raman imaging due to limited out of band rejection (last paragraph on page 85) and which , in addition, is not a filter. Thus, the discussion of LCTFs as cited in Cooney's references both agree that LCTFs at that time were not suitable for Raman chemical imaging. Copies of Cooney's references 14 and 15 have been attached hereto for the Examiner's convenience.

The Applicant therefore respectfully submits that, even with respect to the LCTF disclosed in Cooney, the combination of Cooney and Wach does not disclose a fiberscope having the claimed limitations, mainly that Raman chemical images, Raman spectrographs and optical images are obtained through a common coherent fiber bundle.

CONCLUSION

This invention demonstrates and teaches a fiberscope capable of Raman chemical imaging as well as other important sensing and viewing modes. The Applicant has modified the claims to include limitations wherein the fiberscope can collect Raman chemical images, Raman spectra and video (i.e., optical images) through the same coherent fiber bundle. No single cited reference nor the any combination of Wach, Cooney, Katoot or Alfano disclose the combination as claimed herein. Therefore, the Applicant respectfully submits that the claims, as amended and as discussed above, are patentable and request a Notice of Allowance at the earliest possible time.

Respectfully Submitted,

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APPENDIX

(Marked-Up Versions of Amended Claims.)

2. (Twice amended) A [chemical] Raman imaging fiberscope for the collection of [a chemical image derived from the Raman spectra reflected] white light images, Raman chemical images and Raman spectra from a sample comprising:

an outer jacket;

one or more white light illumination fibers, disposed in said outer jacket, for transmitting white light from a white light source to said sample;

one or more laser illumination fibers, disposed in said outer jacket, for transmitting laser light of a specific laser excitation wavelength from a [first] laser source to said sample;

a coherent fiber bundle, disposed in said outer jacket, for transmitting a [clear] white light image of said sample and a Raman chemical image of said sample based on light scattered, reflected or emitted from said sample from one end of said fiber bundle proximate said sample to the opposite end of said fiber bundle distal said sample;

a [spectral] laser bandpass filter positioned between said one or more laser illumination fibers and said sample for transmitting said laser light of said specific laser excitation wavelength and rejecting light of other wavelengths;

a [spectral] laser rejection filter positioned between said sample and said coherent fiber bundle for transmitting wavelengths of light other than said specific laser excitation wavelength[.];

wherein said white light image, said Raman chemical images and said Raman spectra are all collected through said coherent fiber bundle.

3. (Amended) The [chemical] Raman imaging fiberscope of claim [1] 2 wherein said [spectral] laser bandpass and said laser rejection filters exhibit environmental insensitivity to temperature and humidity.

4. (Twice amended) The [chemical] Raman imaging fiberscope of claim [1] 2 further comprising one or more lenses positioned between said sample and said coherent fiber bundle [for focusing said image on said coherent fiber bundle].

5. (Amended) The [chemical] Raman imaging fiberscope of claim [1] 2 [further comprising a housing for enclosing the fiberscope] wherein said laser bandpass and said laser rejection filters are metal oxide dielectric filters.

7. (Amended) The [chemical] Raman imaging fiberscope of claim 6 wherein said window is composed of a material selected from a group comprising quartz, diamond and sapphire.

6. (Amended) The [chemical] Raman imaging fiberscope of claim [5] 2 further comprising [a] an optically transparent window disposed at the [distal] end of said [fiberscope] outer jacket proximate said sample.

8. (Amended) The [Raman imaging] fiberscope [assembly] of claim [1] 2 wherein said laser [spectral] bandpass filter is spatially patterned into a first portion for filtering said laser light and a second, transparent portion [for transmitting light scattered, reflected or emitted by said sample to said coherent fiber bundle].

18. (Twice amended) [A chemical] The Raman imaging fiberscope [for the collection of a chemical image derived from the Raman spectra reflected from a sample] of claim 2 further comprising:

[one or more laser illumination fibers for transmitting laser light of a specific laser excitation wavelength from a first source to said sample;

a coherent fiber bundle capable of transmitting a clear image of said sample based on light scattered, reflected or emitted from said sample;

a spectral filter positioned between said one or more laser illumination fibers and said sample for transmitting said laser light of a specific laser excitation wavelength and rejecting light other wavelengths;

a spectral filter positioned between said sample and said coherent fiber bundle for transmitting wavelengths of light other than said specific laser excitation wavelength;

one or more lenses positioned between said sample and said coherent fiber bundle;

a spatial filter positioned between said sample and said coherent fiber bundle for controlling the angular field of view of said coherent fiber bundle;

a housing for enclosing said fiberscope; and

a window disposed at the distal end of said fiberscope.]

a liquid crystal tunable filter imaging spectrometer coupled to the distal end of said coherent fiber bundle;

wherein said Raman chemical images are collected by tuning said liquid crystal tunable filter over a range of wavelengths and collecting images for each of said tuned wavelengths over a plurality of spatial locations on the surface of said sample, said spatial locations corresponding to individual fibers in said coherent fiber bundle.

20. (Twice amended) The [chemical] Raman imaging fiberscope of claim 18 [wherein said spectral filters exhibit environmental insensitivity to temperature and humidity.] further comprising a CCD camera, coupled to the output of said liquid crystal tunable filter imaging spectrometer, for viewing said Raman images.

21. (Twice amended) A [chemical] Raman imaging fiberscope [for the collection of a chemical image derived from the Raman spectra reflected from a sample] of claim 18 further comprising:

[a coherent fiber bundle capable of transmitting a clear image of said sample based on light scattered, reflected or emitted from said sample;

a spectral filter positioned between said sample and said coherent fiber bundle for transmitting wavelengths of light other than said laser light of a specific laser excitation wavelength;

one or more lenses positioned between said sample and said coherent fiber bundle;

a spatial filter positioned between said sample and said coherent fiber bundle for controlling the angular field of view of said coherent fiber bundle;

one or more white light illumination fibers for transmitting white light from a second light source to said sample;

a housing for enclosing said fiberscope; and

a window disposed at the distal end of said fiberscope.]

a video CCD; and

a video monitor for the viewing of white light images.

22. (Twice amended) A [chemical] Raman imaging fiberscope for the collection of [a chemical image derived from the Raman spectra reflected] white light images, Raman chemical images and Raman spectra from a sample comprising:

an outer jacket;

one or more white light illumination fibers, disposed in said outer jacket, for transmitting white light from a white light source to said sample;

one or more laser illumination fibers, disposed in said outer jacket, for transmitting laser light of a specific laser excitation wavelength from a [first] laser source to said sample;

a coherent fiber bundle, disposed in said outer jacket, for [capable of] transmitting a [clear] white light image of said sample and scattered Raman light from said sample [based on scattered, reflected or emitted from said sample];

a [spectral] laser bandpass filter positioned between said one or more laser illumination fibers and said sample for transmitting said laser light of a specific laser excitation wavelength and rejecting light of other wavelengths;

a laser rejection filter positioned between said sample and said coherent fiber bundle for transmitting wavelengths of light other than said specific laser excitation wavelength; and

a liquid crystal tunable filter imaging spectrometer.

[one or more lenses positioned between said sample and said coherent fiber bundle;

a spatial filter positioned between said sample and said coherent fiber bundle for controlling the angular field of view of said coherent fiber bundle;

one or more white light illumination fibers for transmitting white light from a second light source to said sample;

a housing for enclosing said fiberscope; and

a window disposed at the distal end of said fiberscope.]

23. [A chemical] The Raman imaging fiberscope of claim [1] 22 further comprising:

a mount for holding [the] said fiberscope [distal end] in proximity to said sample;

a link for directing the output of [the] said fiberscope under white light illumination conditions to a video CCD for viewing on a video monitor [live video camera];

a link for directing the output of said fiberscope under laser illumination conditions to a Raman spectrometer; and

a link for directing the output of said fiberscope under laser illumination conditions to [a Raman chemical imaging spectrometer and detector.] said liquid crystal tunable filter imaging spectrometer.

25. (Twice amended) The [system] Raman imaging fiberscope of claim 23 further comprising software and hardware for producing and displaying a Raman chemical image of said sample.

27. (Twice amended) The [chemical] Raman imaging fiberscope of claim [10] 22 further comprising a spatial filter positioned between said sample and said coherent fiber bundle for controlling the angular field of view of said coherent fiber bundle.

28. (Amended) A [chemical] Raman imaging fiberscope for [imaging and] collecting white light images, Raman chemical images and Raman spectra from a sample comprising:

one or more white light illumination fibers for transmitting white light from a white light source to said sample;

one or more laser illumination fibers for transmitting laser light of a specific laser excitation wavelength from a [first] laser source to said sample;

a coherent fiber bundle;

a [spectral] laser bandpass filter positioned between said one or more laser illumination fibers and said sample for transmitting said laser light of a specific laser excitation wavelength and rejecting light of other wavelengths; and

a [spectral] laser rejection filter positioned between said sample and said coherent fiber bundle for transmitting [images comprising] wavelengths of light other than said specific laser excitation wavelength;

wherein said coherent fiber bundle [can be positioned and focused with respect to said sample using light collected by said coherent fiber bundle.] transmits white light images, images composed of Raman scattered light and Raman spectra from a plurality of locations on the surface of said sample corresponding to individual fibers in said coherent fiber bundle.

29. (Amended) The [chemical] Raman imaging fiberscope of claim 28 wherein said [spectral] laser bandpass and said laser rejection filters exhibit environmental insensitivity to temperature and humidity.

30. (Amended) The [chemical] Raman imaging fiberscope of claim 28 further comprising one or more lenses positioned between said sample and said coherent fiber bundle. [for focusing said image on said coherent fiber bundle.]

31. (Amended) The [chemical] Raman imaging fiberscope of claim 28 further comprising [a housing] an outer jacket for enclosing [the] said fiberscope, said outer jacket containing said white light illumination fibers, said laser illumination fibers and said coherent fiber bundle.

32. (Amended) The [chemical] Raman imaging fiberscope of claim 31 further comprising [a] an optically transparent window disposed at the [distal] end of said [fiberscope] outer jacket.

33. (Amended) The [chemical] Raman imaging fiberscope of claim 32 wherein said window is composed of a material selected from a group comprising quartz, diamond and sapphire.

34. (Amended) The Raman imaging fiberscope [assembly] of claim 28 wherein said laser [spectral] bandpass filter is spatially patterned into a first portion for filtering said laser light and a second, transparent portion [for transmitting light scattered, reflected or emitted by said sample to said coherent fiber bundle].

35. (Amended) The Raman imaging fiberscope [assembly] of claim 28 wherein said [spectral] laser bandpass and said laser rejection filters are [composed of a filter type selected from a group comprising dielectric, holographic and rugate spectral filters] metal oxide dielectric filters.

43. (Amended) The [chemical] Raman imaging fiberscope of claim [1] 2 further comprising a spatial filter positioned between said sample and said coherent fiber bundle for controlling the angular field of view of [said collection] the fibers in said coherent fiber bundle.

44. (Amended) The [chemical] Raman imaging fiberscope of claim 28 further comprising a spatial filter positioned between said sample and said collection fibers for controlling the angular field of view of [said collection] the fibers in said coherent fiber bundle.

45. (Amended) [A chemical] The Raman imaging fiberscope of claim 28 further comprising:

a mount for holding [the] said fiberscope [distal end] in proximity to said sample;

a link for directing the output of [the] said fiberscope under white light illumination conditions to a video CCD for viewing on a video monitor [live video camera];

a link for directing the output of said fiberscope under laser illumination conditions to a Raman spectrometer; and

a link for directing the output of said fiberscope under laser illumination conditions to [a Raman chemical imaging spectrometer and detector.] said liquid crystal tunable filer

imaging spectrometer.

47. (Amended) The [system] Raman imaging fiberscope of claim 45 further comprising software and hardware for producing and displaying a Raman chemical image of said sample.

48. (Amended) A [chemical] Raman imaging fiberscope for [imaging and] collecting [Raman spectra] broadband images, Raman chemical images and Raman spectra from a sample comprising:

one or more white light illumination fibers for transmitting white light from a white light source to said sample;

one or more laser illumination fibers for transmitting laser light of a specific laser excitation wavelength from a [first] laser source to said sample;

a coherent fiber bundle;

a [spectral] laser bandpass filter positioned between said one or more laser illumination fibers and said sample for transmitting said laser light of a specific laser excitation wavelength and rejecting light of other wavelengths;

a [spectral] laser rejection filter positioned between said sample and said coherent fiber bundle for transmitting [images comprising] wavelengths of light other than said specific laser excitation wavelength;

one or more lenses positioned between said sample and said coherent fiber bundle;

a spatial filter positioned between said sample and said coherent fiber bundle for controlling the angular field of view of the fibers in said coherent fiber bundle;

[a housing] an outer jacket for enclosing the fiberscope; and

a window disposed at the [distal] end of said [fiberscope] outer jacket[;

wherein said coherent fiber bundle can be positioned and focused with respect to said sample using white light images collected by said coherent fiber bundle].

Utrasensitive Raman and fluorescence imaging using liquid crystal tunable filters

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ABSTRACT

The principles of the single stage and dual stage liquid crystal Fabry-Perot interferometer (FPI) are discussed. A dual stage liquid crystal FPI which has 11-12 cm^{-1} pass band and is electronically tunable is described. The filter is characterized as a component of a light microscope. Its application to Raman microscopy of materials and to fluorescence microscopy of nucleic acid dynamics is demonstrated with examples from the author's laboratory.

1. INTRODUCTION

Band pass interference filters are almost universally used in fluorescence imaging to isolate the emission from the excitation light. Analogous filter-based Raman imaging was proposed early in the history of the Raman microprobe.[1] The band pass and out-of-band rejection limitations of available filter technology were inadequate, and filter-based Raman imaging was largely ignored until recently. Batchelder and co-workers demonstrated that good Raman images could be obtained even from demanding samples with modern narrow band pass dielectric filters.[2] Treado and co-workers introduced the first electronically tunable band pass filter, the acousto-optic tunable filter (AOTF) to Raman imaging.[3] Our own group has developed high throughput (75%) tunable filters based on holographic transmission gratings.4 Recently, H. R. Morris and co-workers[5] demonstrated that Raman images could be obtained with a tunable liquid crystal Lyot filter.

These filter technologies all have limitations for both Raman and fluorescence imaging. Interference filters have limited angle-tuning range. When the filter is operated more than a few degrees from normal incidence, the band pass increases and the clear aperture decreases. The AOTF is essentially a diffraction grating, and introduces blur in the dispersion direction.[5] The resolution of commercial devices, about 50 cm^{-1}, is marginally adequate for Raman spectroscopy. Improved resolution is bought at the cost of increased filter length, with accompanying increased spherical aberration. The demonstrated dual holographic grating filter, which is essentially a mechanically tuned subtractive double monochromator, also has marginally adequate spectral resolution for Raman imaging. The resolution could easily be improved, but with increased physical size and mechanical complexity.

2. THE LIQUID CRYSTAL FABRY-PEROT INTERFEROMETER

Liquid crystal filters potentially avoid the shortcomings of other filter techniques. They are electronically tunable, have a wide clear aperture and are operable over the spectral range 400-2500 nm. The wavelength switching time is fast enough - several milliseconds in most models - for all Raman imaging and for fluorescence imaging at standard video frame rates or slower.

The demonstrated Lyot filter has these liquid crystal filter advantages,[4] although the pass band of about 200 cm^{-1} is too wide for almost all Raman imaging. The Lyot pass band can certainly be reduced, but its inherently low transmission remains a serious limitation of this technology. Thus, there remains a need for a broadly tunable, narrow pass band liquid crystal filter. The dual-stage liquid crystal Fabry-Perot interferometer (FPI), recently introduced by our group, fills this need.[6] Although the liquid crystal FPI is a new device, the FPI has been used by astronomers for spectroscopic imaging for many years.[7]

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2.1. Theory of the liquid crystal Fabry-Perot interferometer

The Fabry-Perot interferometer consists of two partially transparent optical flats separated by either an air gap or a dielectric spacer. The transmission, $I(\lambda)$, is given by equation 1.[8]

$$I(\lambda) = \frac{1}{1 + F\sin^2(\phi/2)} \quad [1]$$

The angle of incidence, θ, the refractive index of the dielectric between the flats, n, and the cavity length, d, govern the phase difference, ϕ, between two successively transmitted beams, according to equation 2.

$$\phi = \frac{4\pi n d \cos\theta}{\lambda} \quad [2]$$

and F is the reflectivity finesse, which is related to the reflectance of the flats, R, by equation 3.

$$F = \frac{4R}{(1-R)^2} \quad [3]$$

The pass band, γ, of the FPI is given by equation 4.

$$\gamma = \frac{4}{\sqrt{F}} \quad [4]$$

The finesse, F, defines the free spectral range of the interferometer, according to equation 5.

$$F = \frac{\pi\sqrt{F}}{2} \quad [5]$$

The interferometer can be tuned by varying n or d, because a phase change between successive reflections is proportional to the optical path length, nd, within the cavity. Mechanically changing d is common, but an attractive alternative is to change the refractive index, n.. If the cavity is filled with a nematic liquid crystal, then the refractive index can be changed with application of a low AC voltage.

2.2. Some properties of the liquid crystal Fabry-Perot interferometer

A nematic liquid crystal is a birefringent material, whose orientation is a function of the applied voltage. Consequently, a liquid crystal filter requires linearly polarized light as input. If the incident light is randomly polarized, the maximum transmission will be 50%. The Lyot filter contains multiple polarizers, and the transmission is consequently low. The FPI needs only one polarizer and can achieve close to the 50% theoretical transmission. Of course, if the incident light is partially or completely polarized, then the filter transmission can be greater than 50%.

Although the FPI has excellent spectral resolution, it also has limited free spectral range. Consequently, the FPI must be used in tandem with some sort of order-sorting filter for most spectroscopic or imaging applications, including Raman and fluorescence imaging. A second FPI of slightly greater pass band can be used as the order sorter. The dual stage FPI will have a narrow pass band and a surprisingly wide free spectral range, because the transmission is low at any wavelength at

which either stage has low transmission. In the liquid crystal implementation, both FPI stages are tuned synchronously, to yield an electronically tunable band pass filter.

The liquid crystal FPI - or any liquid crystal filter - can have a large clear aperture because it is constructed of polished and coated glass or quartz flats with a thin layer of liquid crystal between them. A clear aperture of 12-15 mm is routine, and larger apertures are available. The large clear aperture facilitates incorporation of a liquid crystal filter into instruments such as microscopes without vignetting.

In principle, an FPI is operable anywhere in the electromagnetic spectrum. In practice, the operating wavelength range is limited by the materials of which the interferometer is constructed. As optical elements liquid crystals are usable from about 400 nm to about 2500 nm. The range is limited by their UV and IR absorption. Glass and quartz are satisfactory optical materials over this range. Typically, plastic film polarizers are employed in liquid crystal filters operating in the visible. They operate over a limited wavelength range. The dielectric coatings of the FPI flats are achromatic only over a limited wavelength range, as well. Either the polarizer or the coatings will limit the range of operation of a liquid crystal FPI.

3. EXPERIMENTAL

The Raman/fluorescence microscope incorporating a dual-stage liquid crystal FPI[6] (Meadowlark Optics, Longmont, CO) is shown schematically as Figure 1. The filter was designed and the flats were coated for operation over the range 550-850 nm. The microscope was an infinity-corrected research microscope (Olympus, BH-2), fitted with dry and water-immersion objectives. Wide-field uniform laser illumination is provided by passage of the laser through a mechanically vibrated multi-mode optical fiber.[9] Typically, 5 mW of laser power (532 nm) was used for fluorescence imaging and 40-100 mW for Raman imaging.

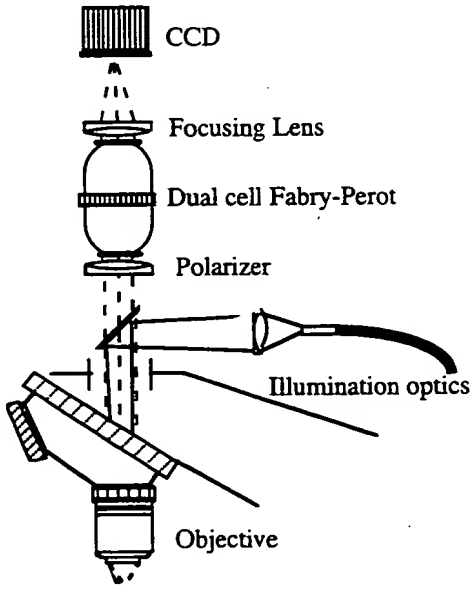

Figure 1. Raman/fluorescence microscope with dual-stage liquid crystal Fabry-Perot interferometer. The illumination source is a frequency-doubled CW Nd-YAG laser (not shown). Auxiliary band rejection filters are not shown.

Raman images were acquired with a Photometrics slow-scan CCD camera, containing a Photometrics PM-512 sensor operated at -130° C. Fluorescence images were acquired with this camera or with a Photometrics Star I slow-scan CCD operated at -50°C. For Raman imaging, one or two Kaiser

Optical Systems holographic super-notch filters were placed in series with the FPI to provide adequate rejection of laser light.

Nucleic acid images were obtained in a locally-constructed electrophoresis cell.[10] Briefly, this apparatus consisted of a microscope slide with platinum electrodes cemented to it, to allow imposition of electric fields. The active region of the slide was covered with a 0.17 mm cover slip, which was cemented to the edges of the slide to prevent evaporation. Nucleic acids (PPFG marker, 225 kbp - 1.9 Mbp, New England Biolabs) were stained with ethidium homodimer I. Electrophoresis was performed in buffers containing 50%-60% (w/w) sucrose to slow nucleic acid motions to the time scale appropriate to the available CCD camera.

4. RESULTS AND DISCUSSION

4.1. Performance of the dual stage liquid crystal Fabry-Perot interferometer

The band width of the dual stage liquid crystal FPI is illustrated by Figure 2, which shows the response as the instrument is tuned through the 730 line of a neon discharge lamp. The band pass of the FPI (FWHM) is 11 cm^{-1}.

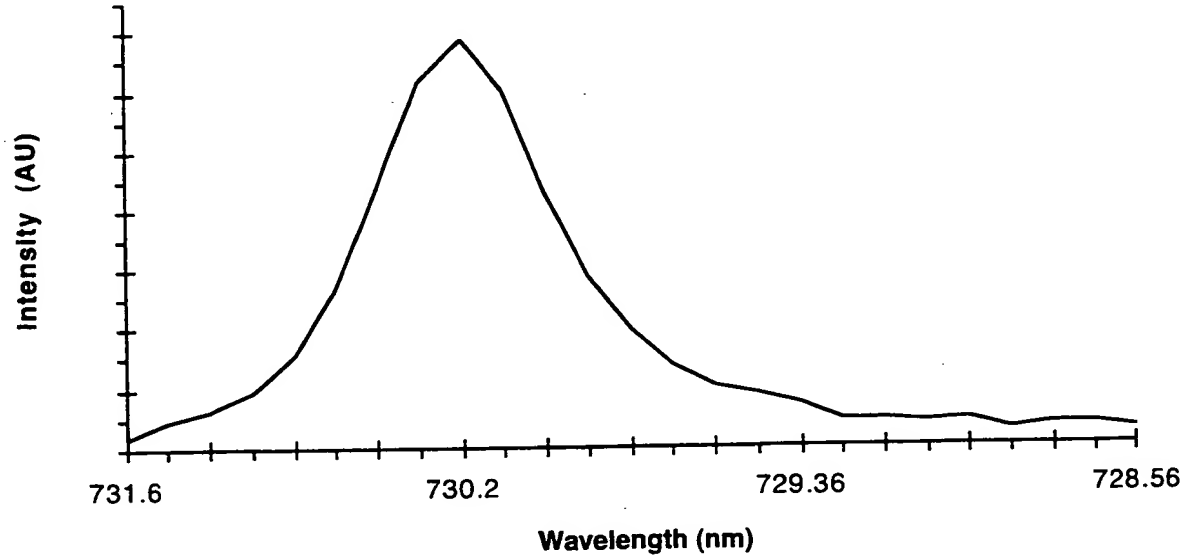

Figure 2. Transmission curve of the dual-stage liquid crystal Fabry-Perot interferometer in the 730 nm region. The light source is the collimated output of a neon discharge lamp.

In the 600-700 nm region the band pass is 11-12 cm^{-1}, i.e. constant to within experimental error. Near the 550 nm cut-off of the dielectric coatings on the flats, the band pass increases as the coating reflectance decreases.[6] The free spectral range is about 45 nm. This relatively small free spectral range is a consequence of the large optical path length difference between the two stages of the filter. The out-of-band rejection of the filter is about 10^3X. This rejection compares favorably with the performance of dielectric filters and AOTF's and is adequate for many fluorescence applications. For Raman imaging, however, additional laser line rejection is needed. One or two stages of holographic notch filtering was found to be necessary for our applications.

Figure 3 shows the bright-field transmission image of a portion of a USAF 1951 bar target taken with the filter in the optical system. Insertion of the filter in the optical system slightly reduces the resolution of the microscope (95%±2% modulation at 228 lp/mm), compared to its performance with the filter out of the system (97%±1%). The source is probably uncompensated spherical aberration from the four interferometer flats in the optical path. Noticeably absent, however, is the blur along one axis which is characteristic of filters based on single diffraction gratings, such as the AOTF.[5]

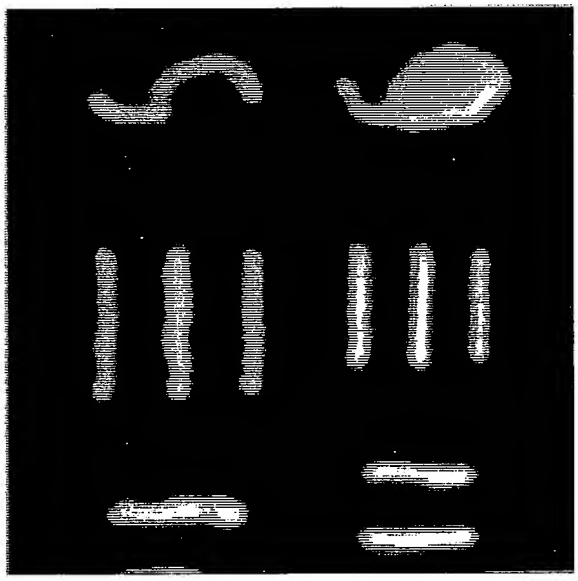

Figure 3. Bright field transmission image of a portion of a USAF 1951 resolution target with dual-stage Fabry-Perot interferometer in the microscope light path.

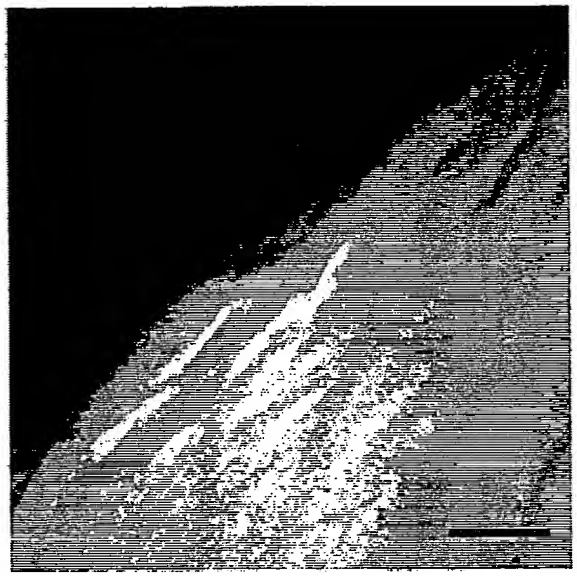

Figure 4. Raman microscope image of polyethylene taken through 20X/0.4 N.A. objective. Excitation 532 nm; scattering, 1457 cm^{-1}. Scale bar = 10 μm.

4. 2. Raman microscopy.

A typical Raman microscope image obtained through the filter is shown as Figure 4. The specimen is a chip of polyethylene imaged at the 1457 cm^{-1} band. The weak fluorescence background was measured at 1510 cm^{-1} and has been subtracted from the image. The specimen was prepared by cutting with a scalpel, and striations in the image are the result of this process.

An important emerging application area for Raman imaging is mapping of active ingredients in pharmaceutical formulations. Here the gross composition of the formulation is known, but the uniformity of the distribution of the active ingredient is frequently uncertain. We have prepared an ascorbic acid/dextrose tablet simulant, which is representative of difficult problems of this kind. We have used low magnification Raman microscopy to map the distribution of ascorbic acid. Typically, high magnification is not needed or even desirable in this application. The ball mill mixing yields particles which are coarse (5-50 μm diameter). The finished tablet may be as large as 1 cm in its largest dimension.

The vitamin C simulant was chosen because both compounds look quite similar under the light microscope. The reflectance image, Figure 5 left, shows the locations of large and small particles, but gives no information on what they are. The Raman image, on the other hand, shows the distribution of ascorbic acid content clearly.

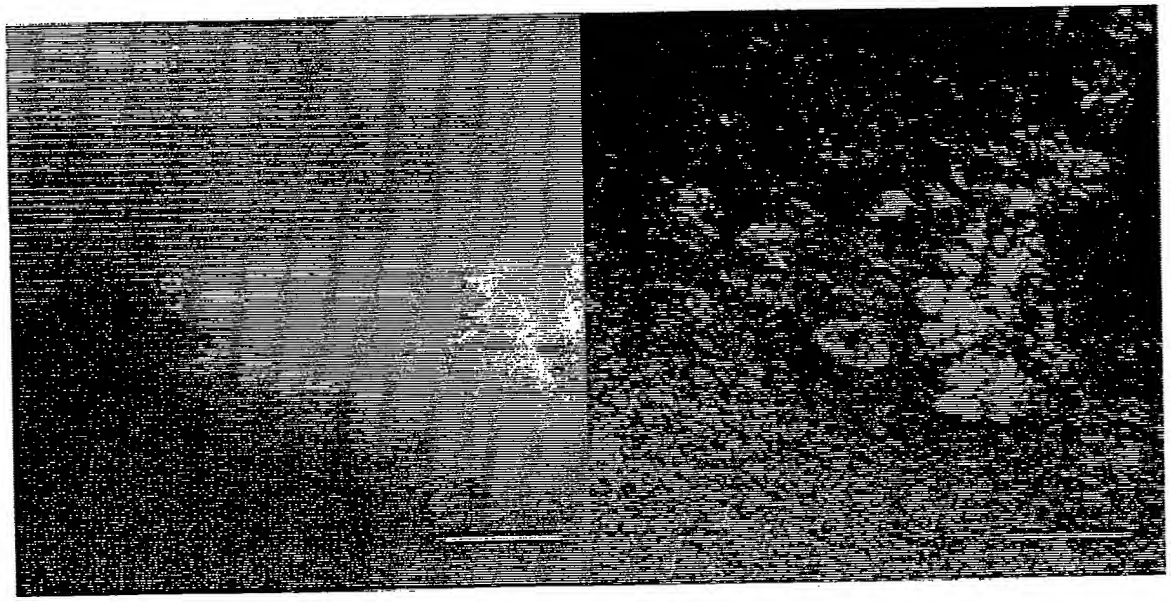

Figure 5. Left. Reflectance image of ascorbic acid/dextrose tablet simulant, 5X 0.13NA objective. Right. Ascorbic acid Raman image at 1667 cm^{-1}, using 532 nm excitation, 5X 0.13NA objective. Scale bar = 200 μm.

The chemical state specificity of vibrational spectroscopy is good, but not perfect, and its limitations are well-known.[11] In many cases it is adequate to image at one Raman band for each mapped constituent, whether or not the band is a classical group frequency. In some cases, such as some polymer blends, there may be no frequency at which the spectra of the components are completely disentangled. In that case, standard multivariate procedures can be used.[12] We have found that principal components analysis with coordinate rotation is an effective procedure which yields resolved images of polyethylene and polystyrene in a blend. However, generation of accurate

components requires use a large spectral interval, and is more practical in spectrograph or interferometer-based imaging than in filter-based imaging. Filter-based Raman imaging is generally faster than spectrograph-based imaging, and certainly requires much less image storage space. However, there will be some cases where more reliable information is obtainable by acquisition of extended spectral intervals at each pixel, followed by processing of the multispectral images.

4.3. Fluorescence microscopy.

The liquid crystal filter is not limited to Raman microscopy, of course. It can function equally well in fluorescence or any other spectroscopic imaging. In condensed phase electronic spectroscopy, 10-20 cm^{-1} band widths are rarely necessary. The filter is useful primarily for its electronic tunability and image fidelity. Figure 6 is a typical example of the images obtainable.

The left-hand frame is one of a sequence of fifteen serial sections taken through the fiber sample at 1 μm intervals. The right-hand frame is the thick section obtained from thirteen images processed with a simple nearest-neighbors deblurring algorithm using a 45% subtraction. The section consists the maximum intensity at each pixel from each of the thirteen deblurred sections, i.e.. $I(x,y) = \max[I(x_i,y_i)],i=\{1,2,...,13\}$.

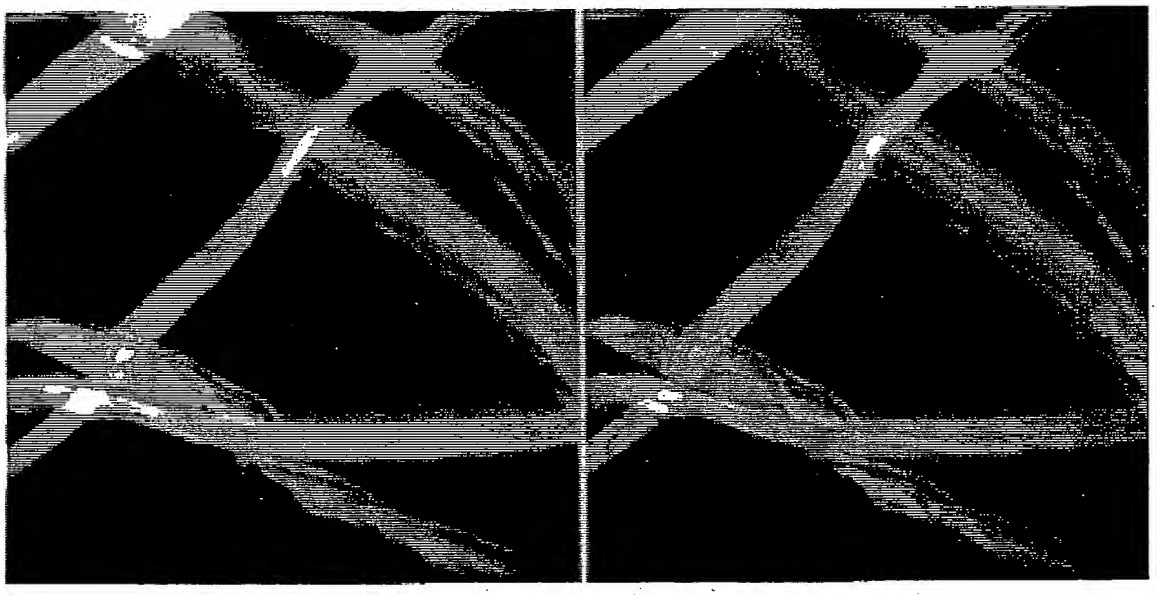

Figure 6. Fluorescence image of cotton fibers, 532 nm excitation, 550 nm emission, 10X/0.3N.A. objective. Left, original image. Right, thick section of thirteen deblurred slices.

4.4. Nucleic acid dynamics.

Over the past five years, fluorescence microscopy has emerged as a major technique for observation of nucleic acid motions during gel electrophoresis. A nucleic acid which is 10^6 base pairs (paired nucleotides) has a contour length of over 300 μm. Segmental motions are readily observable if the nucleic acid is stained with a fluorophore. A typical example of nucleic acid motion during electrophoretic migration in a dilute polymer solution is shown in Figure 7. Because the images were made with a slow-scan CCD, sucrose has been added to the solution to increase the viscosity and stretch the time scale for dynamics.

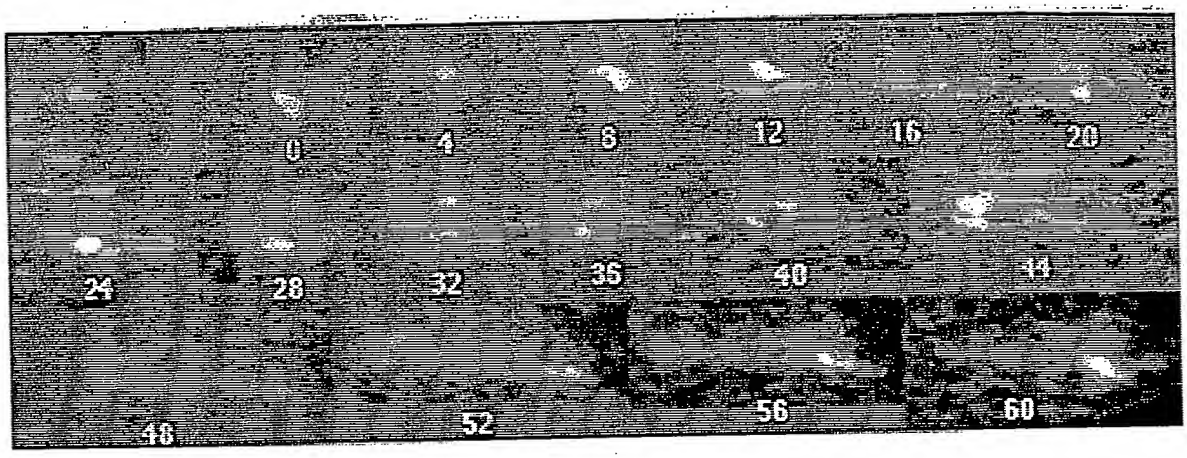

Figure 7. Nucleic acid dynamics. Fluorescence images of stained DNA moving through a 0.045% hydroxyethyl cellulose solution at a driving electric field of 60 V/cm. The images are taken at 4 sec intervals, as marked. The solution contains 58% sucrose to stretch the time scale of nucleic acid segmental motions.

Motions of nucleic acids through dilute solutions of linear polymers have been a special interest of our laboratory. Electrophoretic separations in these media are rapid. We have recently reported separations of megabase-sized DNA in under 15 minutes.[13] The separations work even when the polymers are sufficiently far apart that they can not form an entangled network. The phenomenon contradicts classical electrophoretic theory, and its theoretical explanation is still conjectural.[14] Liquid crystal filters has an important role to play in the elucidation of this form of electrophoresis. Two-color fluorescence imaging allows direct examination of the interaction between nucleic acids and fluorophore-labeled sieving polymers, such as celluloses or dextrans. Correlative imaging is important here, so a filter which can be switched at video frame rates is a useful experimental tool. Similarly, structural detail on the micron scale is important, so the low added aberration of liquid crystal filters makes them especially attractive in this application.

5. CONCLUSIONS

Liquid crystal filters are now a useful technology for multispectral imaging. The tuning rate is fast enough for almost all applications. Several configurations are available, allowing the user to optimize the trade-offs among spectral band width, tuning range, transmission and acceptance angle. The dual-stage Fabry-Perot filter combines narrow band width, wide tuning range and good transmission. The combination of these properties makes it especially suitable for Raman imaging, and for imaging on narrow band width electronic transitions, as well.

6. ACKNOWLEDGMENTS

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